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Fungal biodeterioration accounts for enormous damage to stored material through degradation and the production of secondary metabolites. We are seeking to identify reactions that are essential to the survival of fungi. Those involved in sterol biosynthesis have been shown to be required for cell growth and amenable to control by antifungal compounds. We have shown that interconversion of sterols between free and esterified forms maintains the basic level of free sterols. We have identified two conditional mutants that have a defect in sterol esterification which is attendant to inhibition of cell growth. Additional experiments used mutations in the structural genes for ergosterol biosynthesis to assess their essentiality for the fungi.

Further work under another contract addresses the influence of sterol alterations on the regulation of gene expression in sterol biosynthesis.

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Assessment of the essentiality of *ERG* genes late in ergosterol biosynthesis in *Saccharomyces cerevisiae*

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ABSTRACT

Ergosterol is a large, complex 28-carbon molecule that is a multi-functional component of yeast, and accounts for approximately 2% of the cell's dry weight. Most of the genes in the biosynthetic pathway for ergosterol have been cloned, sequenced, and insertionally inactivated. Strains with null mutations in structural genes late in the biosynthetic pathway are able to grow well on complex laboratory media with glucose as carbon source. It has been presumed that the functions encoded by those genes are unimportant to the organism, and the genes have been called non-essential. Isogenic strains of yeast with insertionally inactivated genes for ergosterol biosynthesis and their allelic wild types have been grown in competition to ascertain any selective advantage for organisms with or without functional ergosterol genes. In every instance tested the wild-type allele afforded a competitive advantage over the isogenic pair with a defect in ergosterol biosynthesis. A general trend was seen in which the earlier in the biosynthetic sequence that a mutation occurred, the less able the strain with that defect was able to compete with the ergosterol-producing organisms. It is concluded that designating these genes as non-essential is specious, conditional, and a consequence of not understanding the various physiological functions of ergosterol in yeast.

INTRODUCTION

Fungi are among the most primitive organisms that synthesize abundant sterols. The principal sterol of fungi, ergosterol, (Figure 1) is structurally similar to the major plant sterols (e.g. sitosterol) and the animal sterol, cholesterol. The yeast *Saccharomyces cerevisiae* is an ideal model organism for use in the study of the physiology of fungal sterols through biochemistry, genetics, and molecular biology. Ergosterol has been recognized as a "consensus" sterol, able to carry out a variety of functions in the organism (Parks and Casey 1995). Studies of the terminal reactions in fungal sterol synthesis have been aided

substantially by a series of antifungal agents. A collection of mutants defective in ergosterol synthesis is also available. These mutations and the antifungal agents inhibit specific enzymatic steps in ergosterol formation. The sterol substrate for the inhibited enzyme is rarely accumulated, rather it is modified by subsequent enzymes in the pathway. The resulting sterol is similar in structure to ergosterol, but lacks the modification that is mediated by the blocked reaction. These altered sterols may be unable to fulfill all of the normal cellular functions mediated by ergosterol, such as the role in membrane structure or regulation of sterol biosynthesis (Parks *et al.* 1985; Smith *et al.* 1996). Figure 2 shows the sequence of reactions late in the synthesis of ergosterol, the structural gene designations, and the sites of inhibition of several of the better known antifungal sterol biosynthetic inhibitors.

YPD is a routine rich medium used for culturing *Saccharomyces,* since that medium is generally regarded as sustaining yeast with demanding nutritional requirements. Some of the null sterol mutants, i.e., those with *erg2* (Ashman *et al.* 1991), *erg3* (Arthington *et al.* 1991), *erg4* (Lai *et al.* 1994), *erg5* (Skaggs *et al.* 1996), or *erg6* (McCammon *et al.* 1984; Gaber *et al.* 1989) mutations, are able to grow aerobically without sterol supplementation on YPD medium. The wild-type alleles of those genes have been designated "non-essential" for *Saccharomyces*. Mutations in other "essential" genes in ergosterol biosynthesis do not permit growth of the organisms, unless cultured anaerobically and with sterol additions to the medium (Parks and Casey 1995). The designation essential or non-essential is ambiguous, however, and is dependent on the composition of the growth medium. *ERG24* has been labeled essential because it is required for aerobic growth on YPD (Lorenz and Parks 1992). However, strains with the *erg24* mutation grow well aerobically on the chemically-defined synthetic complete medium (SC), even without sterol supplementation; under those conditions

ERG24 would be designated as non-essential (Crowley *et al.* 1996). In addition, strains lacking Erg3p function can grow well on YPD with various carbon sources, but can not grow on defined medium with glycerol as the sole carbon and energy source (Smith and Parks 1993). The conditionality of the designation "essential" raises concerns as to the importance of ergosterol biosynthesis to the physiology of *Saccharomyces cerevisiae*.

A test for the relative importance of the structural genes in ergosterol biosynthesis would be to determine if there is a survival advantage of the wild type or the mutant with a defect in ergosterol biosynthesis in a direct competition experiment. If a gene were indeed "non-essential", mutations leading to a loss of that function may cause the mutant to grow better than the wild type. In this paper we describe experiments comparing the selective advantage of yeast having wild-type alleles of various structural genes in ergosterol biosynthesis with isogenic strains having insertionally inactivated ergosterol biosynthetic genes. Our results show that in every case that was examined, the strain with the wild-type gene had a substantial competitive advantage over its counterpart with the mutant allele. Our results also demonstrate the importance of ergosterol to cells in adapting to new growth conditions.

MATERIALS AND METHODS

The yeast extract, peptone, glucose, potassium hydroxide, succinic acid, YNB (yeast nitrogen base without amino acids), Bacto-agar and all organic solvents were purchased from Fisher Scientific. Amino acids and polyethylene glycol (PEG) were obtained from Sigma. Restriction enzymes were purchased from New England Biolabs. The primers were synthesized by Integrated DNA Technologies, Inc.

Media and Growth Conditions:

Strains, except CJ340, were grown on YPD containing 0.5% yeast extract, 1% peptone and either 2 or 5% glucose. CJ340 was grown on synthetic complete (SC) medium with 2% glucose (Rose *et al.* 1990), buffered with succinic acid, and adjusted to pH 5.5 with KOH pellets. Selective defined media were made using the same recipe as SC, adding only necessary amino acids. Spore and prespore media were made as described (Rose *et al.* 1990). Bacto-agar to 2% was added for solid media. All strains were grown aerobically at 30°C.

Strains and their construction:

Strains are listed in Table 1. Strains LPY27 (*erg2*), LPY25 (*erg3*), LPY30 (*erg5*), LPY11 (*erg6*), and CJ340 (*erg24*), discussed in this paper are isogenic (MATa *leu2 his3 ura3-52)* except for the ergosterol mutations. WA10-3-1A, SY13, and BKY48-5C were crossed to LPY7 or LPY9 to make LPY27, LPY25, and LPY11, respectively. LPY7 was constructed by crossing JC530 with 463-1D, and LPY9 was constructed by crossing JC482 with 463-1C. Genetic crosses were performed and tetrad analyses were done by the procedure of Rose *et al.*, 1990. Each construction was assayed for the sterol defect by determination of the specific sterol content (see below).

Strain LPY30, containing the *erg5::LEU2* null allele, was isolated according to the micro-homology mediated PCR targeting technique of Manivasakam *et al.*, 1995. A 42 base homologous oligonucleotide was made based on the -207 to -165 region upstream of the *ERG5* gene (Skaggs *et al.* 1996). An additional 46 base primer was made to the +892 to +938 region just downstream of the stop codon for *ERG5*. Each primer contained 20 bases of the corresponding ends of the *LEU2* gene, and these two primers were used in a polymerase chain reaction with the *LEU2* gene of YEp351 (Hill *et al.* 1986) serving as the template. The

PCR product was then transformed into a diploid, LPY15 (see Table 1), and resulted in a one-step disruption (Rothstein 1991). The sterol profile of strain LPY30 was consistent with a disrupted sterol C-22 desaturase, and a Southern blot confirmed the disruption of the *ERG5* gene.

Growth experiments:

Cultures used for obtaining growth curves were grown to stationary phase, and the cell number was determined by direct count using a hemacytometer. Next, the strains were inoculated into 5 ml of media, in triplicate. Growth was monitored using a Klett-Summerson photoelectric colorimeter, with a green filter.

Competition Scheme:

A diagram showing the competition scheme, a series of transfers and platings at specific time points, is shown in Figure 3. First, a mutant and wild-type strain were grown separately for 48 hours in 5 ml of media and then the cell density of the stationary cultures was determined. The initial competition flask was inoculated at a cell number ratio of 1:1 (wild type:mutant), and an initial plating was done to confirm that the cultures were inoculated at an equal ratio. Every 24 hours a sample of the culture was taken, diluted and plated on solid YPD, except for strains containing *ERG24* and *erg24::LEU2* which were plated on SC medium. Following incubation to allow colony development, the colonies were transferred to media lacking leucine and the ratio of wild type to mutant was determined. Every 48 hours an aliquot of the stationary culture was taken and transferred to fresh media.

The sterol compositions of the isogenic strains with sterol mutations and the wild type were determined using alkaline saponification (Parks *et al.* 1985) and gas chromatography (conditions as described in Fenner and Parks 1989).

RESULTS AND DISCUSSION

Competition between strains with *LEU2* or *leu2*:

The first competition was a control experiment using two isogenic strains, differing only in the *LEU2* nutritional marker. In order for the competition protocol to be valid, it was necessary to establish that a leucine auxotroph (LPY7) could grow as well as the otherwise isogenic leucine prototroph (SY110). LPY7, the sterol wild type, is auxotrophic for leucine, and the ergosterol mutant strains that were used in these experiments are leucine prototrophs. The sterol biosynthetic genes were insertionally inactivated with the *LEU2* gene, providing a way to distinguish between the mutant and wild type. The control competition between strains LPY7 and SY110 followed the same protocol (see Materials and Methods and below) as the succeeding experiments with strains containing sterol alterations. The competition was done on YPD containing 2% glucose. After a period of 166 hours (and three sequential transfers to new media), the ratio of leucine auxotrophs to prototrophs remained the same as during initial inoculation, 1:1. This established that the genetic background, differing only in the *LEU2* allele, did not affect the cell growth phases or generation times of the isogenic strains, and that the leucine nutritional marker could be used as the primary selection for the strains carrying the insertionally inactivated sterol biosynthetic gene.

Effect of glucose concentration on the growth of strains containing *ERG6* and *erg6::LEU2*:

Growth curves were obtained, using LPY11 (*erg6*) and LPY7 (*ERG6*), from experiments in which the concentrations of glucose ranged from 2% to 20%. Since yeast are principally found in natural environments with high solute concentrations, the effect of such hypertonic conditions on the two strains was investigated. As the concentration of glucose

increased, the wild-type strain had slightly longer lag times, causing the culture to enter the exponential growth phase later (Figure 4). There also was a more gradual shift to stationary phase after exponential growth as the glucose concentration increased. The mutant strain experienced similar but much more exaggerated shifts in the culture cycle. The difference can be seen by examination of the plotted growth data. On 2% glucose the wild type was already in exponential growth at the first sampling time of 10 hours, whereas the mutant did not start exponential growth until several hours later. At the highest glucose concentration used, 20%, the wild type entered exponential growth after 12 hours, while the mutant took almost 20 hours. At increasing glucose concentrations, entry to the exponential phase was progressively delayed in both cultures, but to a greater extent in the mutant. Additionally, the shift to stationary phase was less abrupt in the mutant. The mutant experienced greater difficulty in initiating new growth when transferred to fresh media; it also initiated growth retardation earlier, prior to stationary phase. Thus, the strain producing ergosterol made these transitions more easily than did the isogenic sterol mutant.

Competition Experiments:

The routine culturing of yeast generally occurs in media containing 2% or 5% glucose, so these concentrations were used in the experiments described here. As discussed above, control experiments were performed to be certain that the leucine nutritional marker provided no selective advantage or disadvantage to the strains during the experiment.

The experimental protocol is mentioned below and shown diagrammatically in Figure 3. The sterol wild type and allelic mutant of interest were mixed at a 1:1 numerical ratio and inoculated to fresh media. Growth proceeded into the stationary phase and an inoculum was removed to start a second culture. The cultures were sampled at intervals and the ratio of wild type to sterol mutant was determined. Figure 5 shows a single culture cycle

for the wild type and its isogenic mutant strains (panels A, B, C, D, and E). The results of the competition experiments are shown in the adjacent panels (F, G, H, I, J), and represent at least two complete culture cycles sequentially. For clarity and convenience the results are discussed using the sterol genotype rather than the strain name.

For the *ERG6* and *erg6::LEU2* pair, the results are in panels A and F. The extended lag phase was again apparent in the mutant. The generation rates were virtually identical for the two strains during active exponential growth. The competition data (Figure 5F) revealed that the ratio of *ERG6/erg6* steadily increased in both glucose concentrations, but changed much more rapidly in 5% glucose. At 144 hours, the ratio was 10:1 on 2% glucose and 80:1 on 5%.

The culture cycles of the strains containing either the *ERG3* or *erg3::LEU2* strains were more similar (Figure 5B) than was seen for *ERG6* and *erg6::LEU2*, yet the competitive advantage afforded *ERG3* was dramatic. The mutant entered exponential growth two hours after the wild type. The glucose concentration appeared to have little effect on the growth rate of either the mutant or the wild type. The exponential growth rates and times of entry into the stationary phase were similar. However, looking at the competition results from time zero, the *ERG3/erg3* ratio increased on both 2% and 5% glucose (Figure 5G). On the lower sugar concentration, the wild type out-numbered the mutant 12:1 at 72 hours, and on 5% glucose the ratio reached 70:1 in the same time interval.

A very pronounced lag was seen in the *erg2::LEU2* strain in comparison to its isogenic *ERG2* strain. While in the exponential phase, the growth rate of the *erg2::LEU2* strain on both concentrations of glucose was virtually identical to the *ERG2* strain (Figure

5C). The ratio of *ERG2/erg2* increased rapidly on 5% glucose, and after 72 hours the ratio was 100:1 (Figure 5H).

The *erg24::LEU2* mutant cannot grow on YPD with either concentration of glucose, however, it grows well in defined SC media (Crowley *et al.* 1996). The basis for this growth defect on rich media remains unknown. The growth curves and competition experiment were determined in the SC medium with 2% glucose. The mutant spent longer in lag phase and the growth rate was slower than for the wild type (Figure 5D). When the mutant strain was entering exponential growth, the wild-type strain was reaching the end of the exponential phase. Even when the *erg24::LEU2* strain finally entered stationary phase, approximately 15 hours later than the wild type, the culture never achieved the same final density as the wild type. Therefore, the competition results (Figure 5I), showing that the ratio of *ERG24/erg24* increased rapidly from 1:1 at zero time to 80:1 at 96 hours, was not surprising.

The *erg5* mutation appeared to be less deleterious to the growth of *Saccharomyces* in laboratory media than were the others that we have studied here. The *erg5::LEU2* and wild-type cultures presented similar growth curves (Figure 5E). Only the 5% glucose is shown here. Over a period of 120 hours, the *ERG5/erg5* ratio changed slightly, reaching only 1.4/1 (Figure 5J).

From the growth curves and the competition data, it is clear that the production of ergosterol provides a competitive advantage over strains that produce altered sterols because of defects in the biosynthetic pathway. The most obvious difference was seen in the organisms ability to adapt to new and changing growth conditions. This has little consequence, when studying pure cultures on laboratory media, but could be very significant in nature where cultures are subjected to frequent feast/famine cycles. In the laboratory, the

selection of spontaneous revertants in cultures of the sterol mutants would not be uniform. It might be anticipated that *erg5* would be the least susceptible to being overwhelmed rapidly by revertants than would some of the other mutants. *ERG5* is the structural gene for the penultimate transformation in the synthesis of ergosterol (Figure 2). That reaction was also probably one of the last to develop evolutionarily (Bloch 1988). Although the absence of this desaturase appeared to have minimal effect under the experimental conditions reported here, it might be anticipated that other competitive conditions might afford substantial advantage to strains with *ERG5* as opposed to those with the allelic mutant.

Ergosterol generally accounts for 1 to 2% of the dry weight of yeast, although reports of up to 10% have been made (Parks 1978). Economy in the synthesis of such a constituent could effect substantial advantage to an organism. It is apparent that although some ergosterol genes are designated non-essential, the production of ergosterol is important to the competitiveness of yeast. Sterol structural genes were originally labeled non-essential because mutants with insertionally inactivated *ERG* genes were able to grow on routine laboratory media. Yet, it is clear that both the designations of essential, in the case of *ERG24*, and non-essential, for the other genes that have been studied here, are conditional. By contrast, it would be untenable to designate *TRP1*, or a variety of other genes for biosynthetic functions, as non-essential, even though organisms without the *TRP1* mandated functions can grow well on YPD or with tryptophan supplementation. It is difficult to imagine that the function specified by *ERG6* is unimportant to the yeast. This is one of the most metabolically expensive reactions in the organism's biochemistry (Parks 1978; Parks and Casey 1995). If it were truly a non-essential function, it is reasonable to expect that organisms lacking that

function would have gained a substantial selective advantage. Our results fail to demonstrate a survival advantage to the strains lacking *ERG6*.

The casual use of the essential/non-essential designation arises because the various functions of ergosterol are not known. Although there is evidence for a variety of functions for ergosterol, the precise physiological roles have not been defined (Rodriguez *et al.* 1985). It is certainly true that sterols plays an important bulk function in membranes. Regulatory functions have now been established where ergosterol *per se* is important (Smith *et al.* 1996). With further research the precise physiological functions of the sterols will be defined and their role in the survival of the organism under varying conditions can be assessed. At that time the designation of essential as opposed to non-essential would be of little practical significance.

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Table 1 Strain List

Strain	Genotype	Source or Reference
463-1C	*MATa leu2 his3 ura3-52 trp1Δ1*	K.Tatchell
463-1D	*MATα leu2 his3 ura3-52 trp1Δ1*	K.Tatchell
JC530	*MATa leu2 his4 ura3-52*	K.Tatchell
JC482	*MATα leu2 his4 ura3-52*	K.Tatchell
LPY7	*MATa leu2 his3 ura3-52*	This study
LPY9	*MATα leu2 his3 ura3-52*	This study
WA10-3-1A	*MATa erg2-4::LEU2 leu2-3,122 his7-2 ura3-52 ade5*	Ashman *et al.* (1991)
LPY27	*MATa erg2-4::LEU2 leu2 his3 ura3-52*	This study
SY13	*MATa erg3::LEU2 leu2 his3 ura3-52 trp1Δ1*	Smith *et al.* (1996)
LPY25	*MATa erg3::LEU2 leu2 his3 ura3-52*	This study
LPY30	*MATa erg5::LEU2 leu2 his3 ura3-52*	This laboratory
BKY48-5C	*MATα erg6::LEU2 leu2 ura3-52*	Gaber *et al.* (1989)
LPY11	*MATa erg6::LEU2 leu2 his3 ura3-52*	This study
CJ340	*MATa erg24::LEU2 leu2 his3 ura3-52*	This laboratory
LPY15	*MATa/MATα leu2/leu2 his3/his3 ura3-52/ura3-52*	This study
SY110	*MATa ura3-52 his3*	This laboratory

Figure Legends

Figure 1. The structures of the most abundant sterols found in A) fungi (ergosterol) B) animals (cholesterol) and C) plants (sitosterol)

Figure 2. The late steps of the ergosterol biosynthetic pathway along with the name of the sterol formed after each enzymatic step. The antifungal compounds, written in bold, indicate which step of the pathway they inhibit.

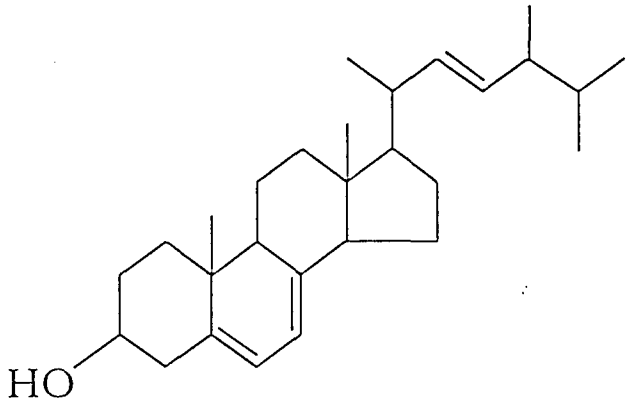
Figure 3. The transfer scheme used during the competition experiments. A mutant and the wild-type strain were grown to stationary phase and then inoculated into fresh media (1:1 ratio). Samples were taken every 24 hours and plated to monitor the competition. Once the culture reached stationary, a sample was taken and placed in fresh media. After 1 or 2 transfers the competition ended and the ratio, calculated every 24 hours, of wild type to mutant was graphed.

Figure 4. Growth curves of A) *ERG6* (–) and B) *erg::LEU2* (- -) over a period of 40 hours on YPD using various concentrations of glucose (•) 2%, (○) 5%, (■) 10%, (□) 15%, and (⋆) 20%.

Figure 5. Growth curves of the ergosterol mutants (- -) and the wild type (–) grown on either 2% (•) or 5% (○) glucose are shown in graphs A-E. A) *ERG6* and *erg6* B) *ERG3* and *erg3* C) *ERG2* and *erg2* were grown on both YPD with 2% and 5% glucose. (D) *ERG24* and *erg24* were grown on SC with 2% glucose, and finally (E) *ERG5* and *erg5* were grown on YPD with 5% glucose. The competition experiments are shown in graphs F-J and the results reported in ratios (wild type/mutant). The theoretical ratio of 1:1 is also plotted (···). *erg6*(F), *erg3*(G), *erg2*(H) were competed on YPD with 2% and 5% glucose;

erg24(I) was competed on SC with 2% glucose; and *erg5*(J) was grown on YPD with 5% glucose.

a.



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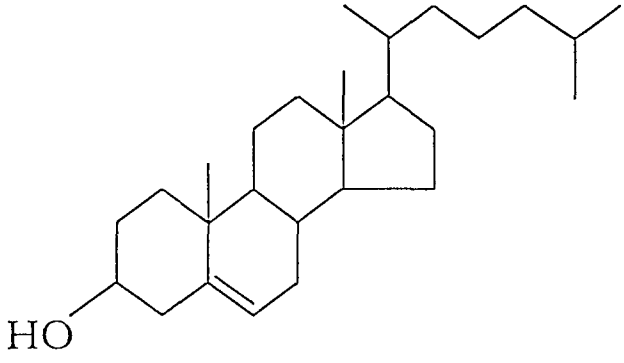

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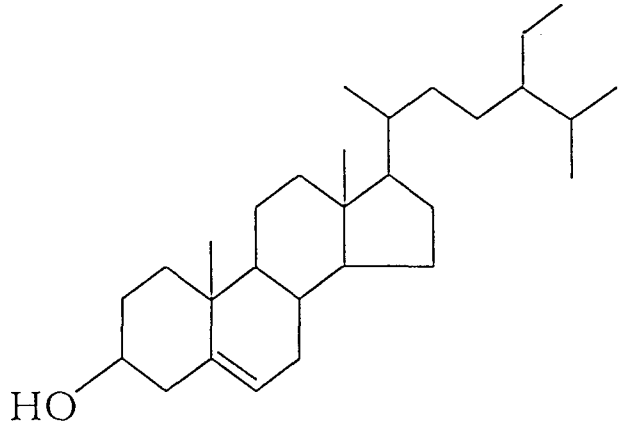


fig 1 - Palermo et al

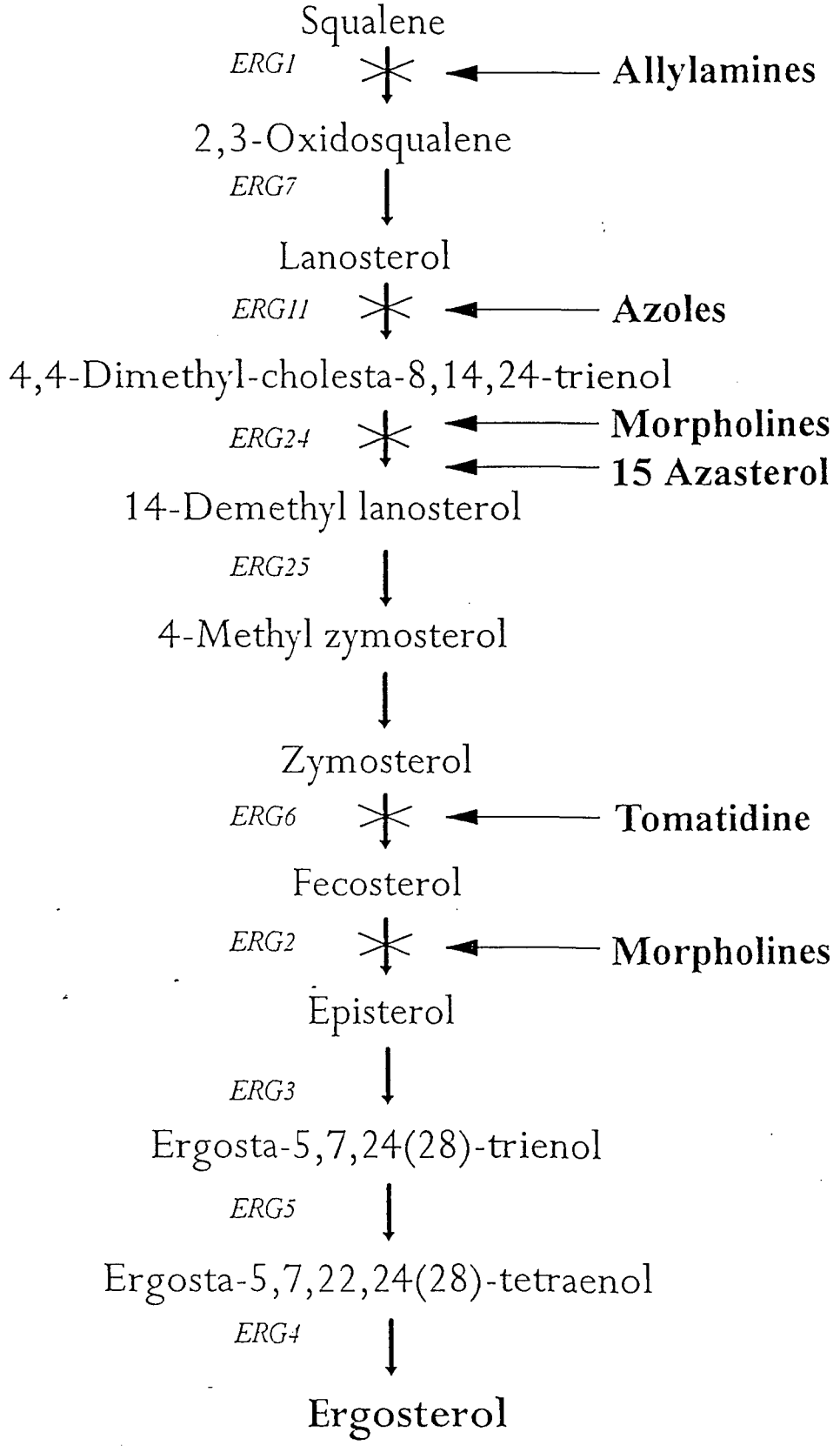


fig 2 - Pallua et a

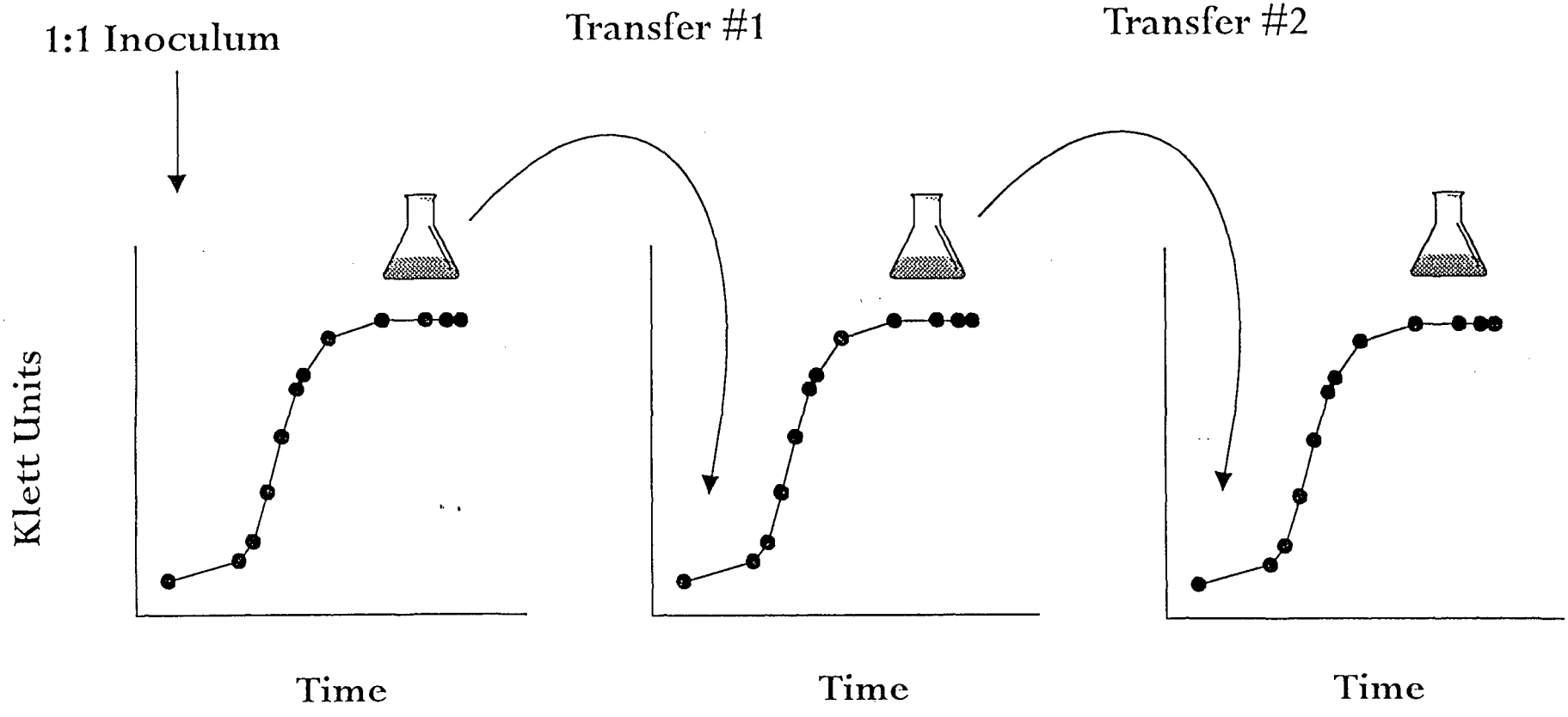


fig 3 - Robertson et al.

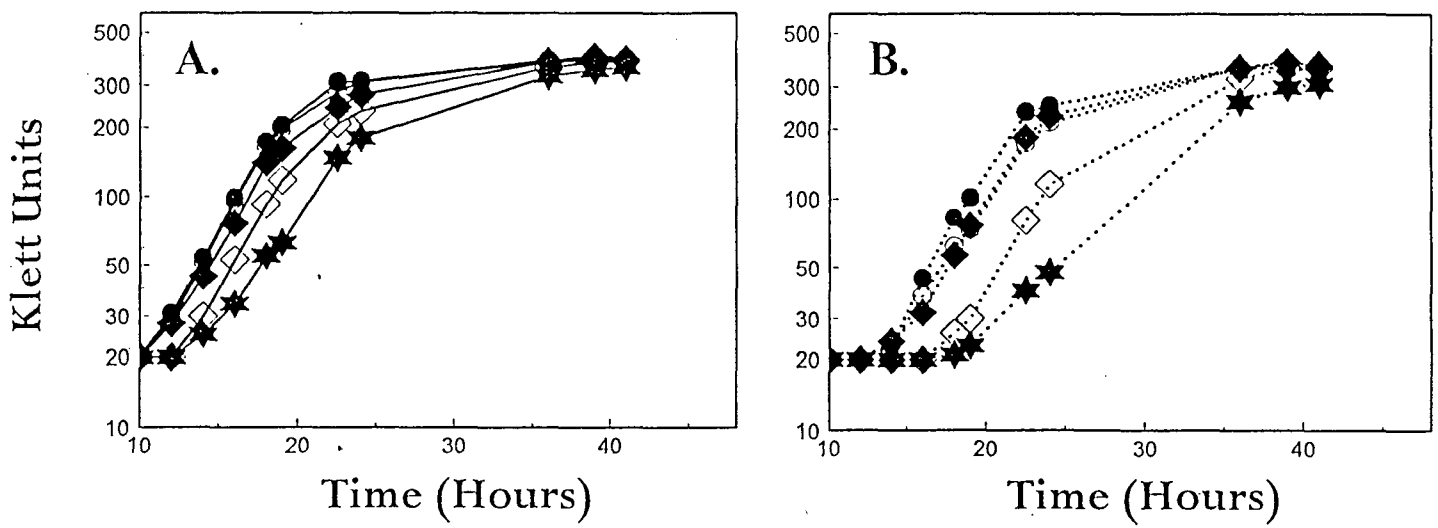
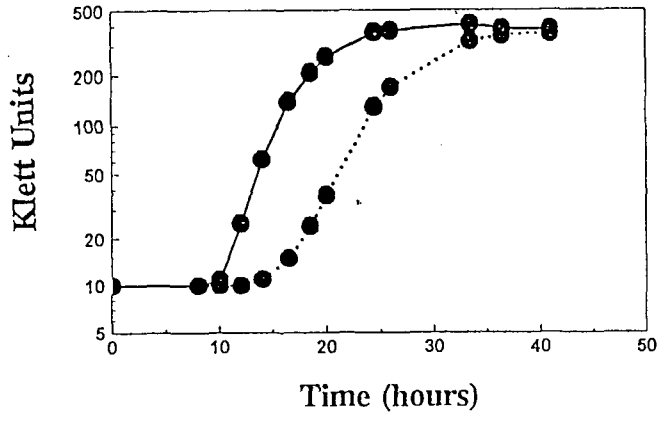
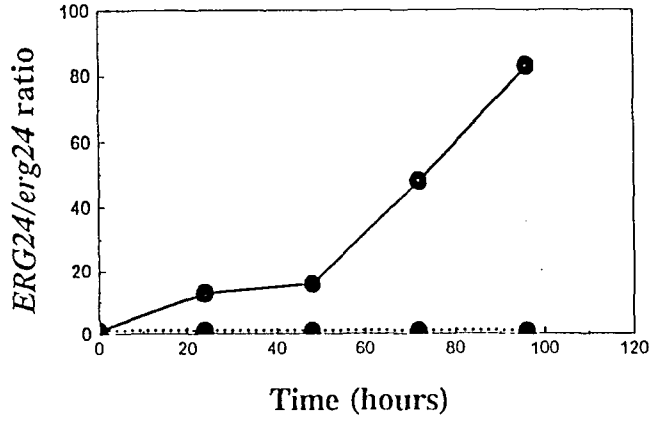


Fig 4 - Reference

D.



I.



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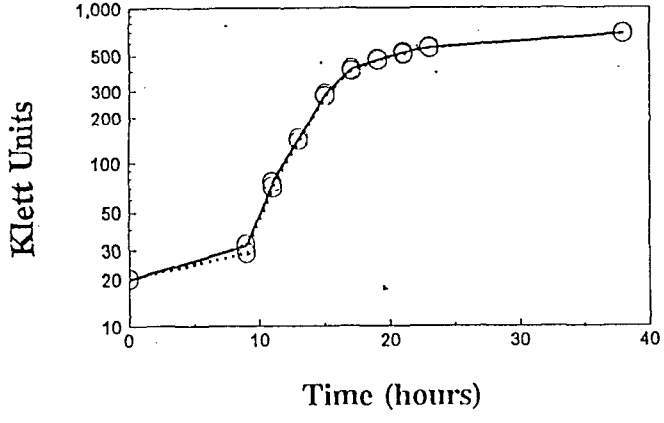

J.

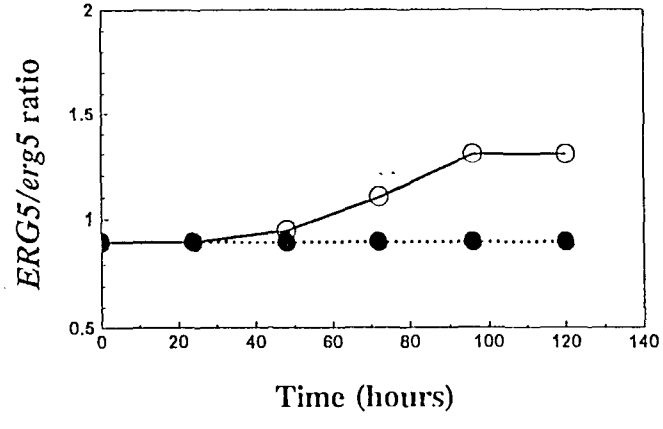

Fig. 5 - Bullard et al.